The Tightly Controlled Deubiquitination Activity of the Human SAGA Complex Differentially Modifies Distinct Gene Regulatory Elements[▽]

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The multisubunit SAGA coactivator complex facilitates access of general transcription factors to DNA through histone acetylation mediated by GCN5. USP22 (*u*biquitin-*s*pecific *p*rotease 22) was recently described as a subunit of the human SAGA complex that removes ubiquitin from monoubiquitinated histone H2B and H2A *in vitro*. Here we demonstrate an allosteric regulation of USP22 through multiple interactions with different domains of other subunits of the SAGA deubiquitination module (ATXN7, ATXN7L3, and ENY2). Downregulation of ATXN7L3 by short hairpin RNA (shRNA) specifically inactivated the SAGA deubiquitination activity, leading to a strong increase of global H2B ubiquitination and a moderate increase of H2A ubiquitination. Thus, SAGA is the major H2Bub deubiquitinase in human cells, and this activity cannot be fully compensated by other deubiquitinases. Here we show that the deubiquitination activity of SAGA is required for full activation of SAGA-dependent inducible genes. Interestingly, the reduction of the SAGA deubiquitination activity and the parallel increase in H2B ubiquitination at inducible target genes before activation do not induce aberrant gene expression. Our data together indicate that different dynamic equilibriums of H2B ubiquitination/deubiquitination are established at different gene regulatory elements and that H2B ubiquitination changes are necessary but not sufficient to trigger parallel activation of gene expression.

Transcription initiation by RNA polymerase II (Pol II) is a highly regulated process that requires the coordinated action of numerous factors. Coactivators are multisubunit complexes that are recruited to promoters by gene-specific activators to facilitate transcription initiation either by direct interaction with general transcription factors and Pol II or indirectly through modifications of the chromatin structure (reviewed in references 18 and 19).

SAGA (*S*pt-*A*da-*G*cn5-*A*cetyltransferase) is a coactivator complex that is functionally and structurally conserved from yeast to mammals and contains two enzymatic activities (14, 32). Histone acetylation is mediated by the catalytic activity of the GCN5 subunit and is thought to facilitate the recruitment of the general transcription machinery (19). Yeast SAGA contains the ubiquitin (Ub)-specific protease (USP) yUbp8, which was shown to remove ubiquitin from monoubiquitinated histone H2B (2, 6). yUbp8 strongly interacts with ySgf73, ySgf11, and ySus1 to from a functional deubiquitination module (DUBm) that is anchored within SAGA through ySgf73 (7, 10, 11, 17, 25). More recently, a similar enzymatic activity carried out by hUSP22 has been described in the human SAGA (hSAGA) complex deubiquitinating monoubiquitinated histones H2A (H2Aub) and H2B (H2Bub) *in vitro* (35–37). Similarly to the yeast complex, hUSP22 was shown to be a component of the DUBm that contains hATXN7, hATXN7L3, and hENY2, the orthologues of ySgf73, ySgf11, and ySus1, respec-

tively. Two ATXN7 paralogues, ATXN7L1 and ATXN7L2, are also components of the hSAGA complex, and due to their strong sequence similarity with ATXN7 it is expected that these proteins have redundant functions with ATXN7 (4, 15). Interestingly, an expansion of a polyglutamine motif found in the N-terminal region of ATXN7 but missing from the other paralogues is responsible for an inherited neurodegenerative disorder called spinocerebellar ataxia type 7 (SCA7) (reviewed in reference 5).

yUbp8 was shown to be inactive unless it is associated with the other proteins of the yeast DUBm. Recent determination of the yeast DUBm structure revealed how this module assembles and activates yUbp8 (12, 29). Similarly to the yeast protein, hUSP22 alone has almost no activity, and we previously hypothesized that hUSP22 has to be incorporated into SAGA to be active (37). hUSP22 and yUbp8 belong to a subclass of USPs that contain a zinc finger (ZnF) domain called ZnF-UBP in their N-terminal region (13, 24). This domain was first characterized as an interactor of free ubiquitin and has been involved in the catalytic activation of USP5, an enzyme which degrades free polyubiquitin chains (27). However, it has been shown that the Ubp8 ZnF-UBP is not able to bind free ubiquitin (7) and sequence conservation analysis revealed that this ubiquitin-binding property is likely absent from USP22 (1). In line with these studies, a mechanism for USP22 catalytic activation that involves the ZnF-UBP domain and its interactions with the other subunits of the DUBm has been proposed (1).

In mammals, monoubiquitination of H2B is catalyzed by the E2 conjugating enzymes HR6A/HR6B and the E3 ligase RNF20/RNF40 (8, 9, 38). The H2B ubiquitination machinery is recruited at the promoter through interactions with activators and is then activated through interactions with additional

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factors such as the PAF complex (34; reviewed in reference 33). However, the precise function of this mark in transcription regulation remains elusive. In mammalian cells, H2Bub was found genome-wide to associate preferentially with transcribed regions of highly expressed genes, suggesting a positive role in transcription (20). In human cells, the SAGA deubiquitination activity is required for full transcriptional activity mediated by nuclear receptors (37). In yeast, increased levels of H2Bub were detected on the GAL1 core promoter and throughout the transcribed region upon transcriptional activation, with both ubiquitination and deubiquitination being required for optimal transcription (6). Hence, H2B monoubiquitination seems to be a highly dynamic process, although its precise functions in transcription regulation remain unclear.

In this study, we show that interactions of USP22 with other proteins of the DUBm are required for its incorporation in the human SAGA complex and for its activation. We also demonstrate that *in vivo*, the SAGA deubiquitination activity is directed against H2Bub and to a lesser extent H2Aub. This activity is required to fully activate SAGA-regulated inducible genes, and our results suggest that this occurs via distinct ubiquitination/deubiquitination cycles at the promoters and the transcribed regions of these genes.

MATERIALS AND METHODS

Plasmids. Plasmids that were used to generate the baculoviruses expressing Fl-USP22, Fl-ATXN7L3, and ENY2 were previously described (37). A full-length USP22 cDNA was PCR amplified using a 5′ primer encoding a hemagglutinin (HA) epitope and cloned into pVL1393 baculovirus transfer vector (BD Biosciences). cDNAs corresponding to ATXN7L3 fragments from amino acid 3 to amino acid 151 [ATXN7L3(3-151)], ATXN7L3(3-76), and ATXN7L3(3-55) were PCR amplified and cloned into a derivative of the pBacPAK8 baculovirus transfer vector (Clontech) containing a sequence encoding a Flag epitope upstream of the cloning site. cDNA corresponding to ATXN7(75-172) and ATXN7(75-142) was amplified by PCR and cloned into a derivative of the pFBDOCS (a kind gift from Imre Berger) containing a sequence encoding the HA and Flag epitopes upstream of the cloning site. Production of recombinant viruses, Sf9 cell infection, and preparation of whole-cell extracts were performed as described previously (37).

Antibodies. The anti-ATXN7L3 (2997) and anti-SPT20 (3006) polyclonal antibodies were obtained by immunization of rabbits with protein fragments corresponding to residues 197 to 276 of human ATXN7L3 and 330 to 531 of mouse SPT20. Antibodies against the following proteins have been described earlier: GCN5 (5GC 2A6), SGF29 (2461), ATXN7L3 (2325), USP22 (2391), ENY2 (2585), and H2B (LG11-2) (15, 22, 37, 21). Other antibodies used in this study are as follows: FLAG (M2 [Sigma]), HA (12CA5), AR (PG21 [Upstate]), RNA Pol II (7G5), H2Bub (NR03 [Medimabs]), H2A (07-146 [Upstate]), H2Aub (E6C5 [Millipore]), and Ub (FK2 [Biomol International]).

Cell culture. HeLa cells were grown in Dulbecco's modified medium supplemented with 1 g/liter glucose, 5% fetal calf serum, and gentamicin. Stress treatments were carried out using 100 nM thapsigargin (Sigma) or dimethyl sulfoxide (DMSO) for 3 h or 4 h. LNCaP cells were grown in RPMI medium supplemented with 10% fetal calf serum, 10 mM HEPES, 1 mM sodium pyruvate, and gentamicin. Before induction, cells were grown in charcoal-treated medium and then treated for 210 min with 10^{-5} M R1881 or with ethanol (EtOH).

shRNA cell line constructions. HEK293T were cotransfected with pLVTH (lentiviral vector containing the short hairpin RNA [shRNA] sequence), pCMVdR8.2, and pMD2G-VSVG plasmids with JetPEI (polyplus transfection). Forty-eight hours later, medium containing lentiviruses was collected and used to infect HeLa or LNCaP cells. Positive cells were sorted by fluorescence-activated cell sorting (FACS). Sequences were as follows: for shATXN7L3, 5′-GGAACTTAGCAAT TCGGATCCTTCAAGAGAGGATCCGAATTGCTAAGTTCC-3′; and for shControl, 5′-CTTACGCTGAGTACTTCGATTCAAGAGATCGAAGTACT CAGCGTAAG-3′.

Complex purifications. Whole-cell extracts from Sf9 cells were immunoprecipitated using anti-HA antibodies coupled to protein A-agarose beads (EZview Red anti-HA affinity gel [Sigma]) for 3 h at 4°C. Antibody-protein A-bound

protein complexes were washed two times with IP buffer (25 mM Tris-HCl, pH 7.9; 10% glycerol; 0.1% NP-40; 1 mM dithiothreitol [DTT]; 5 mM MgCl$_2$) containing 0.5 M KCl and twice with IP buffer containing 100 mM KCl. The immunoprecipitated material was then eluted by peptide competition overnight at 4°C in 100 mM KCl IP buffer. Eluted complexes were analyzed by Coomassie blue staining or Western blotting using the indicated primary antibodies and chemiluminescence detection was performed according to manufacturer's instructions (GE-Healthcare). Whole-cell extracts from HeLa stable cell lines were immunoprecipitated using monoclonal anti-GCN5 (5GC 2A6) and protein G-Sepharose (GE-Healthcare).

***In vitro* deubiquitination assay.** Histones were prepared from HeLa cells by lysing the cells in 10 mM HEPES, pH 7.9; 1.5 mM MgCl$_2$; 10 mM KCl; 0.5 mM DTT; 1.5 mM phenylmethylsulfonyl fluoride (PMSF); 10 mM *N*-ethylmaleimide supplemented with 0.2 M HCl. Samples were incubated on ice for 30 min and centrifuged, and the supernatant was dialyzed first against 0.1 M acetic acid and then against water. Nucleosomes were purified from HeLa cells as described previously (30). Free histones or mononucleosomes were incubated with SAGA complexes immunopurified using an anti-USP22 antibody or purified recombinant USP22 in 100 mM Tris-HCl, pH 8.0; 5% glycerol; 1 mM EDTA; 3 mM DTT for 2 h at 37°C and analyzed by Western blotting using specific antibodies. For *in vitro* assays using ubiquitin vinyl sulfone (Ub-VS [BostonBiochem]), the purified complexes were incubated with 5 μM Ub-VS in a reaction buffer (100 mM Tris-HCl, pH 8.0; 5% glycerol; 100 mM KCl; 3 mM DTT) for 15 min at room temperature (RT). Alternatively, purified complexes were added on mononucleosomes in the presence or absence of 5 μM Ub-VS in a reaction buffer (20 mM Tris-HCl, pH 8.0; 100 mM KCl; 4 mM EDTA; 8 mM MgCl$_2$; 3 mM DTT) for 2 h at 37°C. The reactions were stopped by the addition of Laemmli blue and then the reaction products were analyzed by Western blotting.

RNA isolation, reverse transcription, quantitative PCR (qPCR). Total RNA was isolated using the TRIzol reagent (Invitrogen). Reverse transcription was performed using SuperScript II (Invitrogen) and random hexamers according to the manufacturer's instructions. cDNAs were quantified by real-time PCR using SYBR green PCR master mix (Qiagen or Roche) on a LightCycler 480 instrument (Roche). Primers used are shown at http://igbmc.fr/Lang_mcb2011.

Chromatin immunoprecipitation (ChIP). Cells were cross-linked with 1% formaldehyde at RT for 10 min, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.0], and protease inhibitor cocktail [Roche]), and sonicated using a Bioruptor apparatus (Diagenode) until an average DNA fragment size of 200 to 500 bp was achieved. Supernatants were diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.0) followed by preclearing with sheared salmon sperm DNA, bovine serum albumin (BSA), and protein G-Sepharose (Sigma) (for monoclonal mouse antibodies) or protein A-Sepharose (for polyclonal rabbit antibodies). The precleared chromatin samples were shaken overnight at 4°C with the antibody, and then beads were added for 4 h to the samples to pull down specific protein-DNA complexes. Antibodies used are as follows: RNA Pol II (7G5), H2Bub (NR03 [Medimabs]), H2B (LG11-2), AR (PG21 [Upstate], anti-ATXN7L3 (2997), and anti-SPT20 (3006). The following washes were carried out at 4°C: twice with TSEI (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 150 mM NaCl; 20 mM Tris-HCl, pH 8.0; and protease inhibitor cocktail), twice with buffer TSEII (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 500 mM NaCl; 20 mM Tris-HCl, pH 8.0; and protease inhibitor cocktail), twice with buffer III (250 mM LiCl; 1% NP-40; 1% Na-deoxycholate; 1 mM EDTA; 10 mM Tris, pH 8.0; and protease inhibitor cocktail), and finally twice with Tris-EDTA buffer (10 mM Tris, pH 8.0; 1 mM EDTA). Bound fractions of the chromatin were eluted with 200 μl of elution buffer (50 mM Tris, pH 8.0; 1 mM EDTA; 1% SDS) at 65°C and treated with RNase A (5 μg/ml), cross-link was reversed in 200 mM NaCl at 65°C overnight, and immunopurified material was incubated with proteinase K. DNA was phenol-chloroform extracted, precipitated by ethanol, and then used for qPCR analysis using a Roche LightCycler 480 with SYBR green master mix (Qiagen or Roche). Primers used in the ChIP experiments are available at http://igbmc.fr/Lang_mcb2011.

RESULTS

USP22 enzymatic activity requires its incorporation within the human SAGA complex. Preliminary observations suggested that the free recombinant USP22 has a very weak deubiquitination activity on recombinant histones (36, 37). To test whether free USP22 would have a stronger activity on nucleosomal templates, we directly compared the deubiquitination
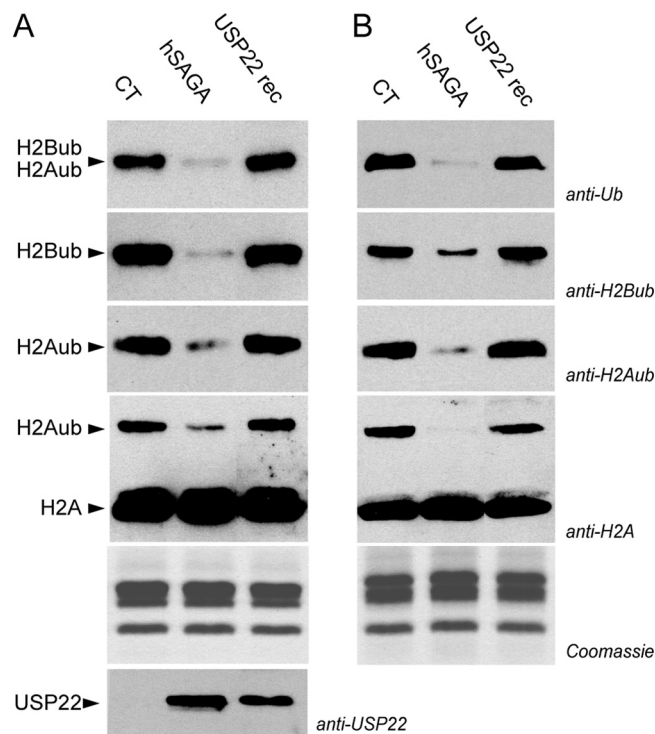
A

B

FIG. 1. H2Aub or H2Bub are deubiquitinated by the purified human SAGA complex but not by the free USP22 enzyme. Mononucleosomes (A) or free histones (B) extracted from HeLa cells and enriched in H2Aub and H2Bub were incubated with no enzyme (CT) or with purified SAGA complex (hSAGA) or recombinant USP22 (USP22 rec). Specific purification of hSAGA complex was verified by mass spectrometry analysis (see Table S1 at http://igbmc.fr/Lang_mcb2011). The deubiquitination reaction was analyzed by Western blotting using antibodies directed against ubiquitin, H2Bub, H2Aub, H2A, and USP22 as indicated. The Coomassie blue staining shows that similar amounts of mononucleosomes or free histones were used.
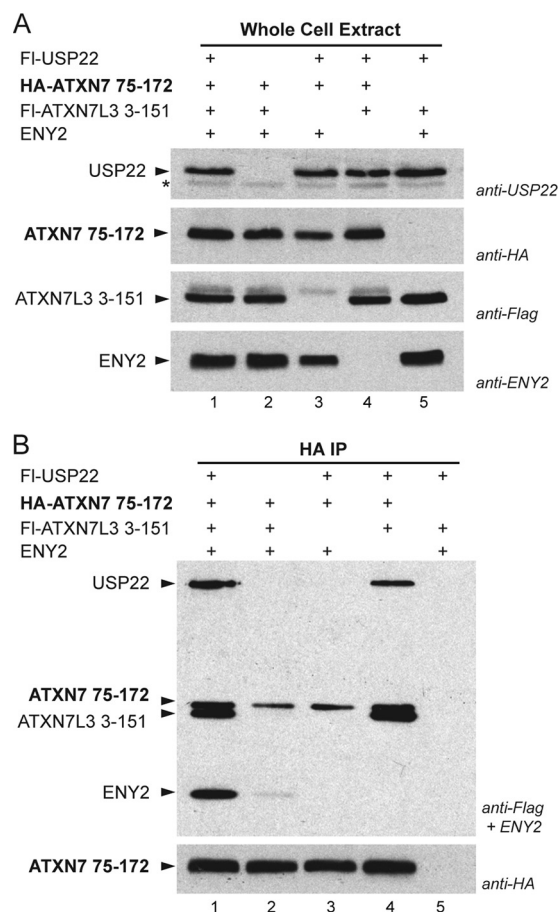
A

B

FIG. 2. The DUB module is stabilized by a network of interactions between the different subunits. (A) Sf9 cells were infected with different combinations of baculoviruses expressing Flag-USP22, HA-ATXN7(75–172), Flag-ATXN7L3(3–151), and ENY2 as indicated. Whole-cell extracts were analyzed by Western blotting and revealed with the indicated antibodies. (B) Immunoprecipitations using an anti-HA antibody followed by peptide elution were analyzed by Western blotting revealed with the indicated antibodies. ATXN7(75–172) (in bold) is harboring the HA epitope. The asterisk indicates a nonspecific band.

activity of the recombinant USP22 with that of the endogenous hSAGA complex after normalization for the amount of USP22 (Fig. 1A, bottom panel) on either free histones (Fig. 1A) or nucleosomes (Fig. 1B) purified from HeLa cells. Although the purified SAGA complex efficiently removed the ubiquitin moiety from H2Aub and H2Bub on histone and nucleosomal substrates, the recombinant USP22 had no detectable activity on the same substrates (Fig. 1). These observations demonstrate that SAGA deubiquitinates H2Aub and H2Bub also in a nucleosomal context and that USP22 has to interact with other SAGA subunits to be fully active.

The human SAGA subunits, ATXN7, ATXN7L3, ENY2, and USP22, assemble in a stable deubiquitination module. To analyze how USP22 gets activated, we first analyzed how the DUBm is assembled. To this end, we coexpressed in Sf9 cells various combinations of the different subunits of the DUBm and immunopurified the corresponding subcomplexes (Fig. 2). In order to study the catalytic regulation of USP22, we analyzed the assembly of the DUBm using a full-length USP22 construct. Furthermore, interaction experiments using truncated versions of USP22 showed that neither the ZnF-UBP domain nor the catalytic domain alone could assemble into a stable complex with ATXN7L3 and ENY2 (see Fig. S1 at

http://igbmc.fr/Lang_mcb2011) (37). We also used a full-length ENY2 construct as this small protein of 101 amino acids is expected from yeast studies to bind a long α helix in the N-terminal region of ATXN7L3 (3). Similarly, we expressed an N-terminal fragment of ATXN7L3 [ATXN7L3(3–151)] that contains the sequence that is homologous to Sgf11, the yeast orthologue of ATXN7L3.

A stable recombinant DUBm could be purified when this ATXN7L3 fragment and the full-length ENY2 and USP22 were coexpressed together with an N-terminal fragment of ATXN7 [ATXN7(75–172)] (Fig. 2B, lane 1). ENY2 appeared dispensable for the DUBm assembly as a complex composed of ATXN7, ATXN7L3, and USP22 could be purified (Fig. 2B, lane 4), even though it appeared slightly less stable than the complex formed with the four proteins. Similarly a stable USP22-ATXN7L3-ENY2 complex was purified in the absence of ATXN7 (Fig. 3B). By contrast, no stable complex could be formed in the absence of either USP22 or ATXN7L3 (Fig. 2B, lanes 2 and 3; also see Fig. S2 at http://igbmc.fr/Lang
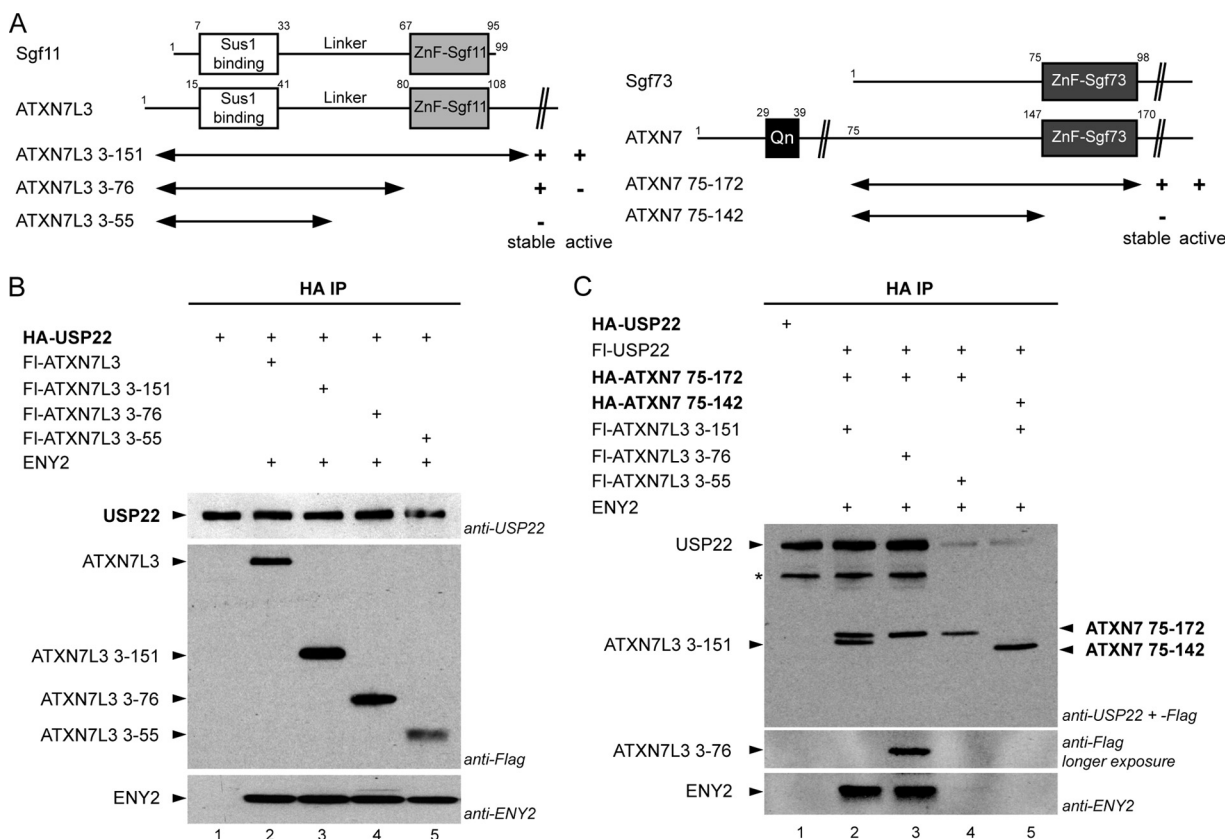
FIG. 3. Identification of the domains required for the formation of a stable DUB module. (A) Schematic representation of the domains of ATXN7L3 and ATXN7 involved in the formation of the DUBm. The functional regions of the human proteins are indicated as compared to their yeast orthologues Sgf11 and Sgf73, respectively (also see Fig. S3 and S4 at http://igbmc.fr/Lang_mcb2011). The different truncated mutants of ATXN7L3 and ATXN7 that were used in immunopurification experiments are shown and their ability to form a stable/active DUBm composed of the four proteins is summarized. (B and C) Sf9 cells were infected with several combinations of baculoviruses expressing different versions of USP22, ATXN7, ATXN7L3, and ENY2 as indicated. Immunoprecipitations using an anti-HA antibody followed by peptide elution were analyzed by Western blotting revealed with the indicated antibodies. Coomassie blue staining of the purified complexes are shown in Fig. S5 at the URL given above. The subunits harboring an HA epitope (that were used to pull down the complexes) are indicated in bold.

_mcb2011). Taken together, these results show that the DUBm is assembled through multiple interactions, rather than pairwise interactions, occurring between the four different subunits of the module.

Determination of the minimal domains required to assemble the deubiquitination module of the human SAGA complex. We next asked which domains of the subunits forming the DUBm are involved in the interaction networks that drive the assembly of this module. Both ATXN7 and ATXN7L3 incorporated within the DUBm through their N-terminal regions, which are composed of their respective ZnF domains (hereafter called ZnF-Sgf73 and ZnF-Sgf11) and poorly conserved N-terminal extensions (Fig. 3A; also see Fig. S3 and S4 at http://igbmc.fr/Lang_mcb2011). Based on this common domain organization of the two proteins, we hypothesized that the ZnF of ATXN7 and ATXN7L3 plays a pivotal role in the assembly and/or the enzymatic regulation of the DUBm.

To test this hypothesis and to investigate the minimal domains of ATXN7L3 that are required to incorporate into the DUBm, we expressed different truncation mutants of ATXN7L3. A mutant containing the whole N-terminal region [ATXN7L3(3–151)] and two mutants deleted for the

ZnF domain [ATXN7L3(3–76) and ATXN7L3(3–55)] interacted with USP22 and ENY2 as well as the full-length ATXN7L3 (Fig. 3B, lanes 2 to 5; also see Fig. S2 at http://igbmc.fr/Lang_mcb2011). In contrast with the smallest mutant [ATXN7L3(3–55)], the ATXN7L3(3–76) mutant, which does not contain the ZnF, could assemble a stable ATXN7-containing DUBm (Fig. 3C, lanes 2 to 4). These results indicate that the ZnF of ATXN7L3 is dispensable for its incorporation into the DUBm. In addition, the "linker" region of ATXN7L3 (amino acids 41 to 80), which is located N terminally from its ZnF-Sgf11 domain, is required for a stable interaction with ATXN7. Finally, the most N-terminal region of ATXN7L3 (Sus1 binding region) (Fig. 3A) bound ENY2 (data not shown), in agreement with the yeast structural data (3).

Similarly, we constructed deletion mutants of ATXN7 to analyze which region mediates its interaction with other subunits of the DUBm. We excluded the most N-terminal low-complexity sequences of ATXN7, which include the polyglutamine motif and are missing from its yeast orthologue, Sgf73, or from human paralogues, ATXN7L1 and ATXN7L2 (Fig. 3A; also see Fig. S4 at http://igbmc.fr/Lang_mcb2011). A mu-

tant containing the ZnF-Sgf73 and the poorly conserved up-
stream sequence [ATXN7(75–172)] stably integrated within
the DUBm, whereas another mutant lacking the ZnF-Sgf73
[ATXN7(75–142)] did not interact with ATXN7L3, USP22, or
ENY2 (Fig. 3C, lanes 2 and 5). The ZnF of ATXN7 is there-
fore required for the assembly of the DUBm, in contrast with
that of ATXN7L3, which is dispensable. This analysis allowed
us to better understand the domains that are necessary to
reconstitute a minimal recombinant DUBm, composed of the
full-length USP22 and ENY2, the N-terminal region of
ATXN7 (amino acids 75 to 172), and the most N-terminal
region of ATXN7L3 (amino acids 3 to 76).

Regulation of USP22 catalytic activity. To understand which
of the domains of the DUBm are required for the activation of
USP22, we analyzed the enzymatic activities of the above-
reconstructed USP22-containing complexes. To this end, we
assessed the catalytic activity of USP22 in the different recom-
binant complexes using ubiquitin vinyl sulfone (Ub-VS), an
irreversible and specific inhibitor of deubiquitinating enzymes.
Ub-VS binds exclusively to an active deubiquitinase but not to
an inactive one. Such binding creates a covalent bound be-
tween ubiquitin and the active site of the deubiquitinase, which
can be revealed by Western blotting as a 7-kDa shift of the
active enzyme corresponding to the size of ubiquitin.

When the free recombinant USP22 was incubated with Ub-
VS, no shift could be detected, indicating that the enzyme
alone is inactive under the assay conditions used (Fig. 4A). We
then treated the purified ATXN7L3(3–151)-ENY2-USP22 and
an ATXN7(75–172)-ATXN7L3(3–151)-ENY2-USP22 recom-
binant complexes with Ub-VS and analyzed the activity of
USP22. USP22 was only partially shifted in the complex con-
taining three subunits but was almost completely shifted in the
complex containing the four subunits (Fig. 4A). These results
together suggest that USP22 is partially activated by its inter-
actions with ATXN7L3 and ENY2 but that its full activity is
also dependent on additional interactions with ATXN7. There-
fore, the full-length ENY2 and the N-terminal fragments of
both ATXN7(75–172) and ATXN7L3(3–151) are sufficient to
incorporate USP22 in a stable and fully active DUBm.

We showed that the ZnF from ATXN7, but not that from
ATXN7L3, is required for the assembly of the DUBm. We
therefore asked whether the ATXN7L3 ZnF plays a role in the
regulation of USP22 enzymatic activity. Using two different
fragments of ATXN7L3 that contain or do not contain the ZnF-
Sgf11 [ATXN7L3(3–151) or ATXN7L3(3–76), respectively], we
purified stable recombinant complexes as above (Fig. 3C) and
tested them in the enzymatic assay using Ub-VS. This analysis
revealed that the ATXN7L3 ZnF-Sgf11 has an additional stimu-
latory effect on USP22 catalytic activity (Fig. 4B).

We further analyzed USP22 activity in the different com-
plexes in another *in vitro* deubiquitination assay using monou-
biquitinated histone H2B, one of the natural substrates of
USP22 (see below). Mononucleosomes purified from HeLa
cells were incubated with different USP22-containing com-
plexes and the levels of H2Bub were analyzed by Western
blotting. Similarly to the results obtained by using Ub-VS, the
free USP22 alone had no detectable activity on H2Bub in this
assay. In contrast, a recombinant DUBm containing the
ATXN7L3(3–151) mutant efficiently deubiquitinated H2Bub.
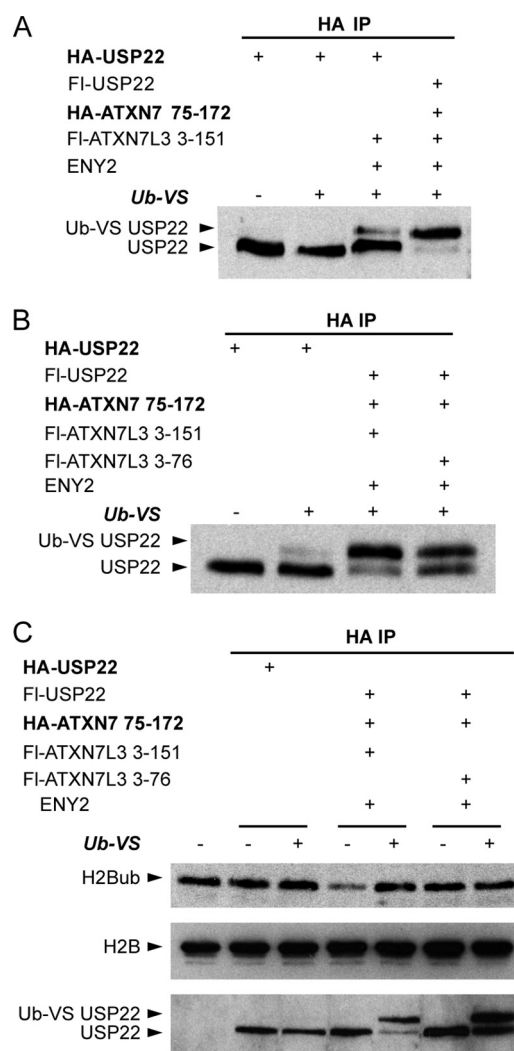This activity was lost when Ub-VS was added to the reaction

FIG. 4. USP22 catalytic activity is regulated by the other subunits
of the DUB module. (A) The DUB module containing the first domain
of ATXN7 is active. (B) The ZnF-Sgf11 of ATXN7L3 is required for
the full activity of USP22. Equal amounts of the different purified
complexes described for Fig. 2 and 3 were incubated with or without 5
μM the suicide substrate ubiquitin vinyl sulfone (Ub-VS). This com-
pound can form a covalent bond only with the active form of USP22,
which is revealed by a 7-kDa shift of the enzyme when analyzed by
Western blotting. (C) The above-described complexes were incubated
with purified mononucleosomes with or without Ub-VS. H2B monou-
biquitination was assessed by Western blotting using an anti-H2Bub
antibody and USP22 reactivity with Ub-VS was measured as described
for panel B.

mixture, further indicating that Ub-VS reacted with the cata-
lytic cysteine of USP22 (Fig. 4C). Interestingly, a complex
containing an ATXN7L3 mutant that lacks the ZnF-Sgf11
[ATXN7L3(3–76)] did not deubiquitinate H2Bub in this
assay. However, the addition of Ub-VS in this reaction induced a
partial shift of USP22 from this latter complex (Fig. 4C). Al-
together, these results suggest that the ATXN7L3 ZnF-Sgf11
plays a role in the allosteric regulation of USP22 but also in the
binding of the SAGA DUBm to nucleosomes. This hypothesis
is strongly supported by structural analysis of the yeast complex
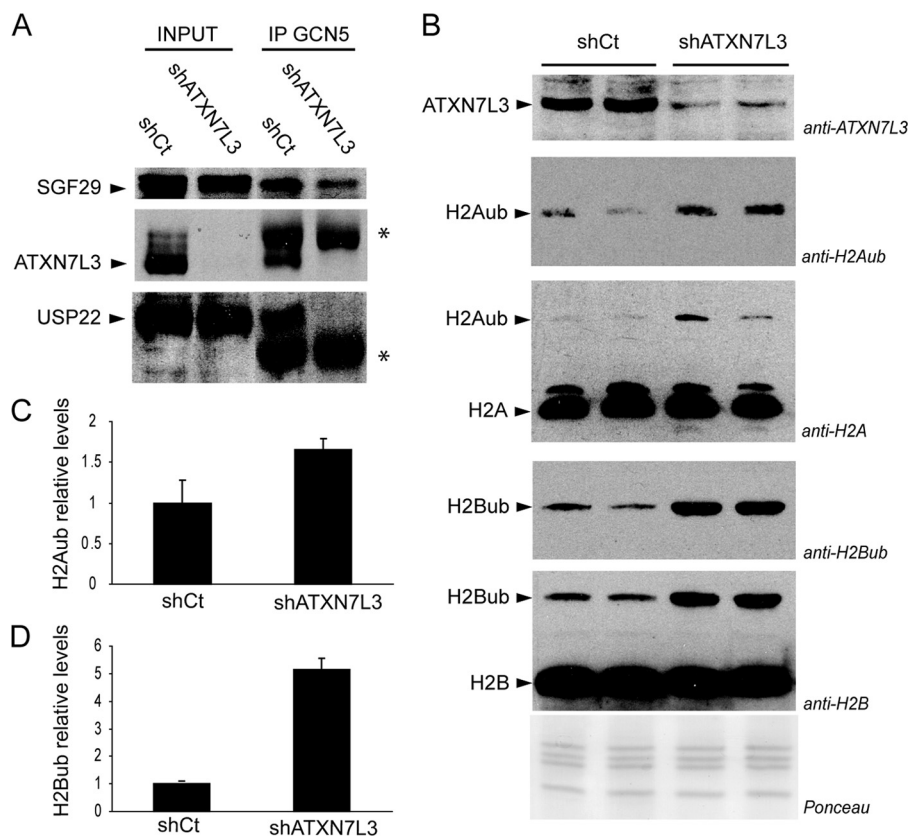showing that the ZnF-Sgf11 of Sgf11 contains a patch of strictly

FIG. 5. Monoubiquitinated H2A and H2B are *in vivo* substrates of hSAGA deubiquitination activity. (A) SAGA complexes were immunopurified from HeLa cells stably expressing a shRNA against ATXN7L3 (shATXN7L3) or a control shRNA (shCt) by using anti-GCN5 antibodies and analyzed by Western blotting with the indicated antibodies. IgG heavy chains are indicated (*). (B) Total histones were purified by acidic extractions from these cell lines. Levels of ubiquitinated histones were analyzed with specific antibodies as indicated. (C and D) H2Aub and H2Bub relative levels were quantified and normalized to H2A and H2B levels, respectively.

conserved positively charged residues, which are exposed to solvent and may be involved in interactions with nucleosomal DNA to target the DUBm to its substrate (12, 29) (see Fig. S3 at http://igbmc.fr/Lang_mcb2011). Thus, these results show an allosteric regulation of USP22 through multiple interactions with different domains of the other subunits of the DUBm including domains that are not necessary for a stable assembly of this module.

***In vivo* deubiquitination activity of the SAGA complex.** Although the human SAGA complex was shown to deubiquitinate H2Aub and H2Bub *in vitro* (35–37), it has not been demonstrated that these substrates are recognized by SAGA *in vivo*. To specifically inactivate the SAGA deubiquitination activity, we generated a HeLa cell line expressing a specific shRNA against ATXN7L3, an hSAGA subunit that is required for both the assembly of the DUBm and the activation of USP22. Immunopurification of GCN5-associated proteins from this cell line, in which the ATXN7L3 levels were strongly reduced, revealed that not only ATXN7L3 but also USP22 was lost from the SAGA complex (Fig. 5A). This result further indicates that ATXN7L3 is crucial for the integrity and thus the activity of the SAGA DUBm, in good agreement with our *in vitro* interaction studies (Fig. 2 and 3).

Next, we asked whether the global levels of monoubiquitinated histones are affected in this cell line, in which the SAGA

deubiquitination activity is impaired. We observed a 1.5-fold increase of H2Aub levels in ATXN7L3 knockdown cells, indicating that human SAGA can participate in the deubiquitination of histone H2A *in vivo* (Fig. 5B and C). Moreover, the global levels of H2Bub were dramatically increased after ATXN7L3 knockdown, indicating that SAGA is a major H2Bub deubiquitinase and that this activity can not be fully compensated for by other deubiquitinases (Fig. 5B and D).

Induction of SAGA-dependent gene expression strongly increased H2B ubiquitination at the transcribed regions but not at the promoters. To further understand the regulation of H2A and H2B ubiquitination in human cells (22, 37), we analyzed the dynamics of these modifications at SAGA-regulated genes in two different cell types. We used LNCaP cells, in which we studied the androgen receptor (AR)-dependent stimulation of the *PSA* gene in the presence or absence of an AR ligand, the dihydrotestosterone analogue R1881 (Fig. 6A and B). We also studied two endoplasmic reticulum (ER) stress genes (*HERPUD* and *ERP70*) in HeLa cells before and following induction of ER stress with thapsigargin (Fig. 7A to C). To measure *in vivo* histone ubiquitination, we used chromatin immunoprecipitation (ChIP) experiments with antibodies recognizing either H2Aub or H2Bub in the two cellular systems. Unfortunately, the commercially available anti-H2Aub anti-
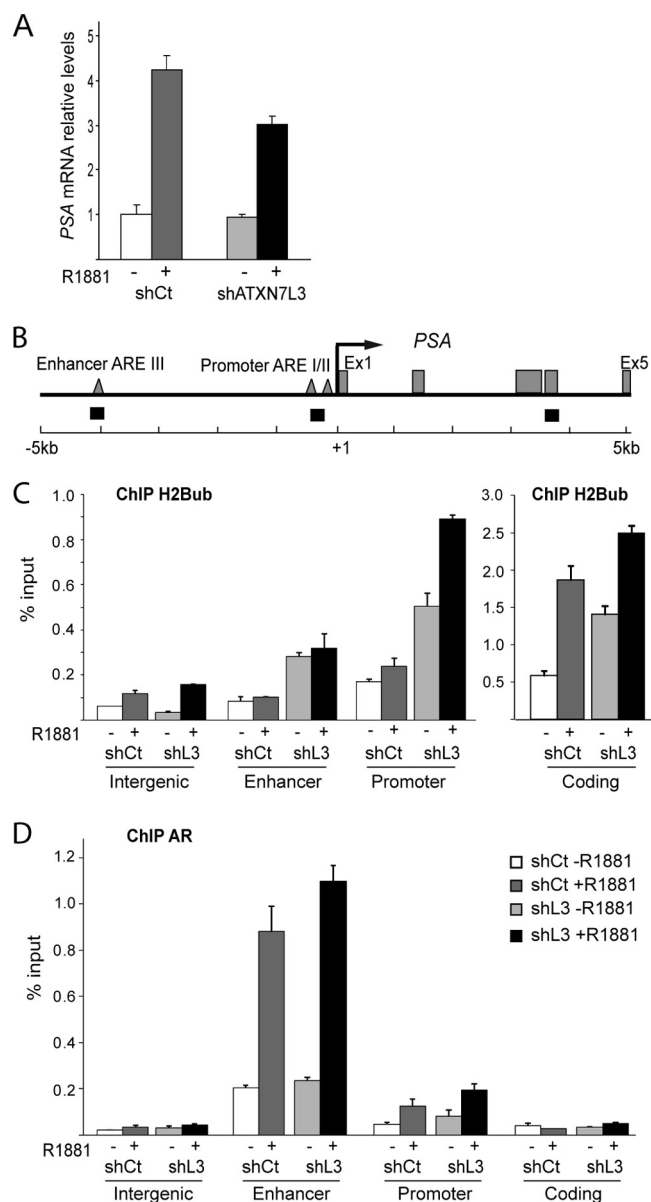
FIG. 6. Regulation of H2B ubiquitination at different regions of an AR-dependent gene. (A) LNCaP cells stably expressing a shRNA against ATXN7L3 or a control shRNA were treated with or without an AR ligand (R1881). mRNA levels of an AR-dependant gene (*PSA*) were analyzed by qPCR of reverse transcribed RNA and normalized to *GAPDH* mRNA levels and are presented as mean values ± SD of three independent experiments. (B) Schematic representation of the genomic locus of the *PSA* gene. Black boxes represent primer sets used for ChIP analysis. (C and D) Control LNCaP cell lines and LNCaP cell lines expressing a shRNA against ATXN7L3 were incubated with (+) or without (−) ligand (R1881) for 210 min. Quantitative ChIP assays were performed for H2Bub (C) and AR (D). Precipitated DNA was quantified by real-time qPCR using primers on enhancer/AREIII, promoter/AREI/II, and coding region/exon4 of the *PSA* gene and an intergenic control region. The values (mean ± SD of triplicates) are expressed as percentages of the amount of immunoprecipitated DNA normalized to the respective input DNA signal (% input). These results are representative of three independent experiments. The same color code is used in all panels.

bodies did not give us reproducible results in ChIP, and thus the dynamics of this modification could not be further studied.

First, we analyzed the distribution of H2Bub at promoters of the *PSA* gene (in LNCaP cells) and of the *HERPUD* and *ERP70* genes (in HeLa cells) before and after induction. Interestingly, H2B ubiquitination seen for the three promoters did not significantly change after induction (Fig. 6C and 7D, compare shCt − and + in promoter panels). At the *PSA* enhancer (AREIII), the H2Bub levels were also unchanged after induction in LNCaP cells, whereas the AR was strongly recruited at this region following induction, as demonstrated by the anti-AR ChIP (Fig. 6D). These results suggest that in wild-type cells following gene activation, the H2B-ubiquitinating enzymes are not active at the enhancer and the tested promoters or that their activity is reversed by a deubiquitination activity at these regions. In favor of the latter hypothesis, our ChIP experiments using antibodies against two different hSAGA subunits (SPT20 and ATXN7L3) revealed an increased recruitment of the SAGA complex at the promoters of *HERPUD* and *ERP70* after induction (Fig. 7F and G).

Next, we analyzed H2B ubiquitination on the transcribed regions of the three genes. Following transcription induction, H2B ubiquitination strongly increased (2.5- to 4-fold) in the transcribed regions of all tested genes (Fig. 6C and 7D, compare shCt − and + in coding panels). These observations are in good agreement with the previously described positive correlation between H2Bub levels and gene expression (20). However, these results strikingly contrast with the observed unchanged H2Bub levels at the promoter regions after induction, suggesting a differential regulation of this modification in the different transcriptional regulatory regions.

The SAGA deubiquitination activity is required for optimal gene expression. As we have shown that SAGA is a major H2Bub deubiquitinase *in vitro* and *in vivo*, we further asked whether this activity influences the regulation of SAGA-dependent inducible gene expression. To this end, we used the above-described shATXN7L3 HeLa cell line and generated an LNCaP cell line in which the SAGA deubiquitination activity was impaired by expressing the shRNA against ATXN7L3. In the two different cellular systems, we observed a 60 to 90% reduction of ATXN7L3 mRNA levels compared to those in control shRNA-expressing cells (see Fig. S6 at http://igbmc.fr /Lang_mcb2011).

Next, we analyzed the expression of the *PSA* gene after treatment with an AR ligand and that of the *HERPUD* and *ERP70* genes after thapsigargin treatment for 3 or 4 h. Following ligand treatment, the *PSA* gene induction was reduced by 20 to 30% in the ATXN7L3 knockdown LNCaP cells (Fig. 6A). Similarly, in the shATXN7L3-expressing HeLa cells, the expressions of *HERPUD* and *ERP70* were reduced by 25% and 45%, respectively (Fig. 7A and B). To demonstrate that the observed differences are explained only by the specific inactivation of the SAGA deubiquitination activity and not by other SAGA functions, we analyzed by ChIP the recruitment of SAGA in this shATXN7L3 cell line. The increased recruitment of ATXN7L3 at the promoters of ER stress genes after induction was lost in ATXN7L3 knockdown cells, further indicating that the used anti-ATXN7L3 is specific. In contrast, the recruitment of the SAGA core at these promoters after induction was not altered by the downregulation of ATXN7L3 as
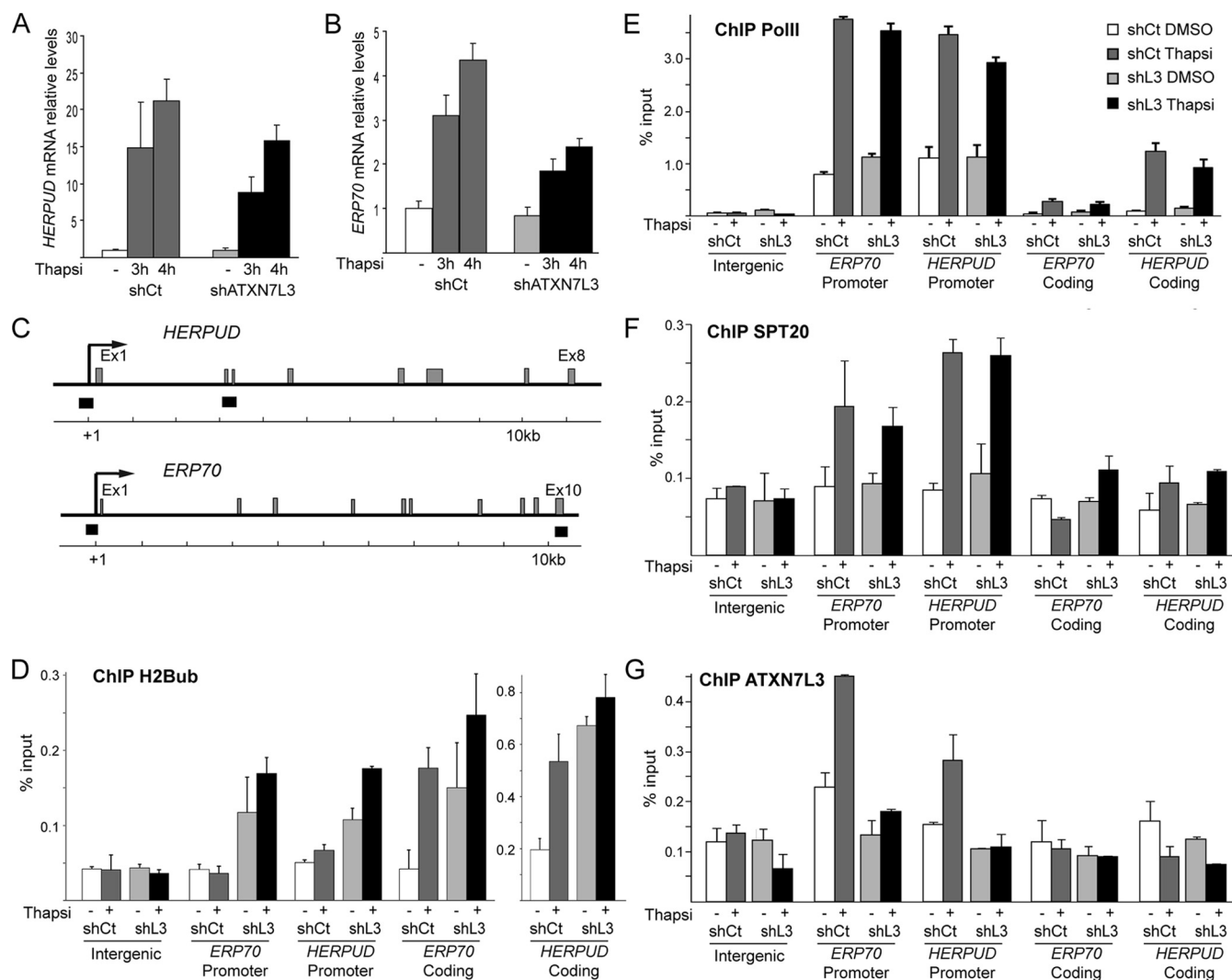
FIG. 7. Dynamics of H2B ubiquitination at the promoter and the transcribed region of ER stress genes. (A) HeLa cells stably expressing a shRNA against ATXN7L3 or a control shRNA were treated with thapsigargin for 3 or 4 h or with DMSO as a control. mRNA levels of ER stress genes (*HERPUD* and *ERP70*) were analyzed by qPCR of reverse transcribed RNA and normalized to *CycloB* mRNA levels and are presented as mean values ± SD of three independent experiments. (B) Schematic representation of the genomic locus of the *HERPUD* and *ERP70* genes. Black boxes represent primer sets used for ChIP analysis. (D to G) Control and ATXN7L3 shRNA HeLa cell lines were incubated with thapsigargin for 4 h or with vehicle only (DMSO). Quantitative ChIP assays were performed for H2Bub (D), RNA Pol II (E), SPT20 (F), and ATXN7L3 (G). Precipitated DNA was quantified by real-time qPCR using primers on *ERP70* and *HERPUD* promoters and coding regions and on an intergenic control region. The values (mean ± SD of triplicates) are expressed as percentages of the amount of immunoprecipitated DNA normalized to the respective input DNA signal (% input). These results are representative of three independent experiments. The same color code is used in all panels.

revealed by a SPT20 ChIP (Fig. 7F and G). These results indicate that the deubiquitination activity associated with the hSAGA complex is required for full activation of different SAGA-dependent genes.

SAGA deubiquitinates H2Bub at the promoter and transcribed regions of SAGA-regulated genes. To understand how the control of H2B ubiquitination by SAGA contributes to the regulation of gene expression, we compared H2Bub levels in ATXN7L3 knockdown cells with those of control cells at the different gene regulatory regions. Surprisingly, our ChIP analyses showed that H2B ubiquitination at the three tested promoters was increased by 2.5- to 3.5-fold in ATXN7L3 knockdown cells before induction when compared to the noninduced control cells (Fig. 6C and 7D, compare shCt − to shL3 − in

promoter panels). The increase of H2Bub levels at promoters when the SAGA deubiquitination activity is impaired reveals the activity of H2B-ubiquitinating enzymes at the promoter region of these genes, even in the absence of induction. Interestingly, this strong increase of H2Bub under noninduced conditions in the ATXN7L3 knockdown cells had no effect on transcription, as we did not measure transcriptional changes from the three studied promoters before induction (Fig. 6A and 7A and B). At these promoters, we observed a further 1.5- to 2-fold increase of H2Bub after induction in the shATXN7L3 cell line as compared to the noninduced conditions. This result demonstrates an increased activity of H2B-ubiquitinating enzymes at promoters following induction of gene expression.

Next, we analyzed the transcribed regions of these genes and

observed that before induction, the H2Bub levels strongly increased (by 3- to 4-fold) in the ATXN7L3 knockdown cells as compared to control cells (Fig. 6C and 7D). This result indicates that SAGA deubiquitinates H2Bub not only at the promoter but also in the transcribed region of these SAGA-dependent genes. In contrast, an increased recruitment of SAGA was observed only at the promoter but not in the gene body of *HERPUD* and *ERP70* upon induction (Fig. 7F and G). Interestingly, the H2Bub increase in the coding regions before induction did not induce aberrant transcription from the studied genes in these cells (Fig. 6A and 7A and B). After induction, H2Bub levels were further increased (by about 1.5-fold) in the ATXN7L3 knockdown cells when compared to control cells (Fig. 6C and Fig. 7D, compare shCt + with shL3 + in coding panels). Concomitantly, transcription activation was reduced by about 25 to 45% (Fig. 6A and 7A and B). However, the recruitment of RNA Pol II at the promoter and the coding regions of *HERPUD* and *ERP70* was not modified after ATXN7L3 knockdown in either induced or noninduced conditions (Fig. 7E).

It is noteworthy that, at each individual region examined in the two cellular systems, the total H2B levels were not significantly changed after ATXN7L3 knockdown (see Fig. S7 at http://igbmc.fr/Lang_mcb2011). However, at the *PSA* enhancer and promoter region, an approximately 2-fold reduction of H2B occupancy could be detected after induction in both control and ATXN7L3 knockdown cells (see Fig. S7 at the URL given above), which likely reflects nucleosome eviction upon transcriptional activation.

In summary, our data indicate that different dynamic equilibriums of H2B ubiquitination are established at different gene regulatory elements. Moreover, our results suggest that at the studied promoter and transcribed regions, changes of H2B ubiquitination alone are not sufficient to trigger parallel changes in gene expression.

DISCUSSION

The allosteric regulation of human USP22. Recent structural and functional studies of the yeast DUBm demonstrated an allosteric regulation of Ubp8 through multiple interactions with other subunits of the DUBm (12, 29). In good agreement, we show here that the enzymatic activity of human USP22 is similarly regulated by its interactions with ATXN7, ATXN7L3, and ENY2. We show that this regulation is mediated not only by domains that are required for the assembly of DUBm but also by the ZnF of ATXN7L3, which is not involved in the assembly of the DUBm. Our results thus suggest an allosteric regulation of human USP22 in which the ZnF domains of ATXN7 and ATXN7L3 play a crucial role. Therefore, the mechanisms of the regulation of SAGA deubiquitination activity appear remarkably conserved through evolution.

Similarly to the yeast complex, the assembly of the human DUBm is mediated through multiple interactions between the four different subunits. These interactions mainly involve the ZnF-UBP of USP22 and the N-terminal regions of ATXN7 and ATXN7L3. However, subtle differences between the yeast and the human complex can be detected. Our reconstruction experiments showed that, in contrast to yeast Ubp8, (12) the ZnF-UBP from USP22 is not sufficient to build up a stable

DUBm (see Fig. S1 at http://igbmc.fr/Lang_mcb2011), suggesting that the protease domain of USP22 is required to stabilize the DUBm. Functional studies of the yeast DUBm revealed that Sgf73 was absolutely required for Ubp8 activation, as an Ubp8-Sgf11-Sus1 complex was inactive (11), whereas in the case of the human DUBm the USP22-ATXN7L3-ENY2 complex was partially active (Fig. 4A). In addition, the N-terminal ZnF-Sgf73 domain of human ATXN7 is required for the assembly of the DUBm (Fig. 3), although the homologue domain in yeast Sgf73 is dispensable (12). Therefore, it appears that structural elements such as the ZnF domain of Sgf73 or the protease domain of Ubp8, which are mainly involved in the enzymatic regulation of the yeast DUBm, have evolved to participate in the stability of the human DUBm in addition to playing their role in the allosteric regulation of the enzyme. This suggests a coevolution of both the stability of the DUBm and the enzymatic regulation of the deubiquitinase, highlighting the biological relevance of the regulation of the deubiquitination activity of SAGA.

The allosteric regulation of USP22 could play an important role in controlling the specific recognition of the substrates by the enzyme. USP22 within the SAGA complex is recruited at specific loci on the genome, where it will act on its specific substrates, H2Aub and H2Bub. Importantly, the mechanism of allosteric regulation described here would prevent the free USP22 enzyme that is not incorporated into the SAGA complex from acting on nonspecific substrates.

The majority of SAGA DUB activity on H2Bub is not redundant with other USPs. A recent study suggested that USP22 might be found in complexes other than SAGA (31), and ENY2 is expected to be part of an mRNA export complex (16, 28). Therefore, the downregulation of USP22 or ENY2 might alter functions other than the SAGA deubiquitination activity. Moreover, human cells contain two additional ATXN7 paralogues (ATXN7L1 and ATXN7L2), which are highly similar with ATXN7 (4) and which could likely compensate for the loss of ATXN7. In contrast, the downregulation of ATXN7L3, which has not been described in any protein complex other than SAGA and whose function is not redundant with that of any other human protein, should exclusively impair the SAGA deubiquitination activity.

Therefore, by comparing H2Bub levels between control cells and cells in which ATXN7L3 is downregulated, we could directly measure where the SAGA deubiquitination activity is acting on different genomic positions and indirectly where the SAGA complex is recruited. The analysis of different inducible SAGA-dependent genes using anti-H2Bub ChIP experiments revealed several unexpected features of the SAGA deubiquitination activity. First, SAGA appears to have a global deubiquitination activity as revealed by the important increase of global H2Bub levels in ATXN7L3 knockdown cells. In agreement, our anti-H2Bub ChIP experiments suggest that SAGA could maintain a low level of H2B ubiquitination at different gene regulatory elements genome-wide. Indeed, even in noninduced conditions, the inactivation of SAGA deubiquitination activity increased the H2Bub levels at the enhancer, promoter, and transcribed regions of the three tested genes. This demonstrates that SAGA regulates H2Bub at different gene regions irrespective of their transcriptional status. However, our ChIP experiments failed to detect stable SAGA recruitment at

these regions in the noninduced conditions, suggesting a very transient and dynamic association of SAGA on these genes. After gene induction, SAGA recruitment was detected only at the tested promoters but not in the transcribed regions. In contrast, the deubiquitination activity of SAGA, as tested by H2Bub ChIP in the shATXN7L3 versus wild-type cells, revealed SAGA activity at both promoter and transcribed regions. These results suggest that after activation, SAGA association with chromatin in the gene body is transient, whereas it is less dynamic at the promoter. Such stable recruitment of SAGA at the promoter could be explained by interactions with promoter-associated activators and histone posttranslational modifications recognized by the different reader domains of SAGA, which in turn would enhance the residency time of SAGA. Finally, the striking difference in H2Bub levels between wild-type and ATXN7L3 knockdown cells indicates that most of the SAGA deubiquitination activity is not redundant with that of other deubiquitinases. Although different H2B deubiquitinases have been identified in yeast and in *Drosophila*, USP3 was the only other human enzyme suggested to deubiquitinate H2Bub (23). However, the precise role of USP3 has still to be determined.

The dynamics of H2B ubiquitination and deubiquitination at promoters and transcribed regions are differentially regulated. Our ChIP analyses on different SAGA-dependent genes showed an increased H2B ubiquitination at the corresponding promoters in ATXN7L3 knockdown cells as compared to control cells, under noninduced conditions. This observation suggests the interesting possibility that the deubiquitination activity of SAGA is already present together with the activities that ubiquitinate H2B at the inducible gene promoters before activation, leading to equilibrium between these two antagonistic activities. Moreover, the lack of changes in transcription under noninduced conditions in the ATXN7L3-downregulated cell line versus wild-type cells suggests that at the studied inducible promoters, the deregulation of the deubiquitination activity and the consequent upregulation of H2B ubiquitination have no direct consequence on transcription.

Our results also show that the SAGA deubiquitination activity can very rapidly reverse the action of H2B-ubiquitinating enzymes at the promoter of SAGA-dependent genes after induction. In yeast cells, it has been shown that the machinery of H2B ubiquitination is recruited to promoters by activators where coactivator complexes such as SAGA are also expected to be recruited through interactions with specific activators. However, the time course recruitment of each complex is unknown, and it is possible to speculate that both complexes are recruited simultaneously by an activator at the promoter of SAGA-dependent genes. In this case, a very fast deubiquitination may be necessary to start efficiently a new cycle of transcription and to maintain a high level of H2B-ubiquitinating enzymes around the promoter. Therefore, a constant cycling of H2B ubiquitination and deubiquitination could be required to maintain the respective enzymes and their associated factors at the promoter of SAGA-dependent genes.

In the transcribed region of these SAGA-dependent genes, the H2Bub levels were significantly increased after induction, suggesting that SAGA is less stably recruited at these regions than at the promoters. Alternatively, much higher levels of H2Bub in the transcribed region could exceed the ability of

SAGA to deubiquitinate H2B. In addition, it has been suggested that H2Bub facilitates the action of FACT, thereby facilitating transcription elongation (26). Our results do not support this model, as in ATXN7L3 knockdown cells in the noninduced conditions, a dramatic increase of H2Bub levels in the transcribed region of SAGA-dependent genes did not correlate with increased transcription of these genes.

Our study highlights the tight control of USP22 catalytic activity through an allosteric mechanism based on complex interactions with other subunits of the SAGA DUBm. Furthermore, our data suggest that SAGA has a global deubiquitination function that appears required to maintain low levels of H2Bub at the promoter and in the transcribed region of genes in noninduced conditions. Our study demonstrates that, after induction, SAGA deubiquitinates H2Bub at both the promoters and the transcribed regions of SAGA-regulated genes but that this complex has very different dynamics on these distinct regions.

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